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SNAP-25 Is Targeted to the Plasma Membrane through a Novel Membrane-binding Domain*

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SNAP-25, syntaxin, and synaptobrevin are SNARE proteins that mediate fusion of synaptic vesicles with the plasma membrane. Membrane attachment of syntaxin and synaptobrevin is achieved through a C-terminal hydrophobic tail, whereas SNAP-25 association with membranes appears to depend upon palmitoylation of cysteine residues located in the center of the molecule. This process requires an intact secretory pathway and is inhibited by brefeldin A. Here we show that the minimal plasma membrane-targeting domain of SNAP-25 maps to residues 85–120. This sequence is both necessary and sufficient to target a heterologous protein to the plasma membrane. Palmitoylation of this domain is sensitive to brefeldin A, suggesting that it uses the same membrane-targeting mechanism as the full-length protein. As expected, the palmitoylated cysteine cluster is present within this domain, but surprisingly, membrane anchoring requires an additional five-amino acid sequence that is highly conserved among SNAP-25 family members. Significantly, the membrane-targeting module coincides with the protease-sensitive stretch (residues 83–120) that connects the two α-helices that SNAP-25 contributes to the four-helix bundle of the synaptic SNARE complex. Our results demonstrate that residues 85–120 of SNAP-25 represent a protein module that is physically and functionally separable from the SNARE complex-forming domains.

Intracellular membrane trafficking depends on specific interactions between vesicles and target membranes. Biochemical and genetic studies demonstrate that integral membrane proteins referred to as SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) are important elements in this process (1, 2). The best characterized SNARE proteins are those that mediate synaptic vesicle exocytosis in nerve terminals (reviewed in Ref. 3). SNAP-25 (synaptosome-associated protein of 25 kDa) and syntaxin are plasma membrane proteins that bind to the synaptic vesicle protein, synaptobrevin/vesicle-associated membrane protein. The critical role that these proteins play in neurotransmission is underscored by the fact that all three are targets of tetanus or botulinum neurotoxins that block neurotransmitter release.

SNAP-25, syntaxin, and synaptobrevin bind to each other to form a stable heterotrimeric complex that consists of a four-helix bundle (4, 5). SNAP-25 contributes two helices from its N- and C-terminal domains; syntaxin and synaptobrevin each provide one. Syntaxin and synaptobrevin are anchored in opposing membranes through transmembrane domains at their C termini. The parallel orientation of these membrane-anchored helices within the complex has led to a model in which the zippering action of complex formation brings the membranes together, with the energy of complex formation driving membrane fusion (6–8). However, whether SNARE proteins directly mediate fusion remains uncertain (9).

Unlike synaptobrevin and syntaxin, SNAP-25 is associated with membranes through palmitoylated cysteines found near the center of the molecule (10). The cysteine-rich sequence is contained within the linker between the N- and C-terminal helices that SNAP-25 contributes to the core synaptic fusion complex (4). The structure of the linker domain is unknown, but is presumed to be exposed and disordered in the complex *in vitro* because of its susceptibility to proteolytic cleavage (11, 12). In any case, this region of SNAP-25 has to be sufficiently extended to accommodate the parallel orientation of both SNAP-25 helices in the synaptic fusion complex (4).

Palmitoylation is one of several lipid modifications that otherwise soluble polypeptides use to associate with the cytoplasmic face of intracellular membranes (reviewed in Ref. 13). SNAP-25, GAP-43 (growth-associated protein of 43 kDa), and cysteine string protein are examples of proteins modified by thioester-linked palmitate at multiple cysteine residues. Other proteins located at the plasma membrane are modified sequentially with two different lipid moieties. These include many non-receptor tyrosine kinases and G-protein α-subunits that are fatty acylated with both amide-linked myristate and thioester-linked palmitate and certain isoforms of Ras that are prenylated and palmitoylated.

The role that different lipid moieties play in the trafficking of newly synthesized proteins to their resident membranes is beginning to be defined. Dual lipidation by myristoylation and palmitoylation appears to confer rapid targeting of $p59^{fyn}$, a non-receptor tyrosine kinase, to the plasma membrane, whereas modification with palmitate alone is associated with slower kinetics of membrane association of GAP-43 and SNAP-25 (14, 15). We demonstrated previously in neuronal cell lines that palmitoylation of SNAP-25 and GAP-43, but not $G_o\alpha$, is sensitive to agents such as BFA[1] that disrupt the secretory pathway (15). These results are consistent with at least two pathways for targeting lipid-modified proteins to the plasma membrane.

Lipidated proteins are typically modified near their N or C termini. SNAP-25 is unusual in this regard with its centrally

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[1] The abbreviations used are: BFA, brefeldin A; GFP, green fluorescent protein; PCR, polymerase chain reaction.

located cysteine cluster. This motif is distinct from the N-terminal palmitoylation motifs found on other neuronal proteins such as GAP-43 and SCG-10. The first 35 amino acids of SCG-10, which include the palmitoylated cysteines at positions 22 and 24, contain the membrane-targeting information (16). Membrane association of GAP-43 is dependent upon palmitoylated cysteines at positions 3 and 4 (17). Previous studies have demonstrated that the palmitoylated cysteines are necessary for membrane association of SNAP-25 (18, 19). However, it has not been determined whether the cysteine-rich domain is sufficient to confer plasma membrane localization. Indeed, we have suggested previously that SNAP-25 may bind to syntaxin (or another protein) to facilitate the subsequent interaction of SNAP-25 with a palmitoyltransferase (15). In this study, we sought to define and characterize the plasma membrane-targeting domain of SNAP-25. Using a deletion and mutagenesis strategy, we mapped the minimal region of SNAP-25 required for membrane localization to a centrally located 35-amino acid sequence. In addition to the palmitoylated cysteines, plasma membrane localization of SNAP-25 also requires a novel five-amino acid motif at the C terminus of this membrane-targeting domain.

EXPERIMENTAL PROCEDURES

Construction of SNAP-25/GFP Fusion Plasmids—The SNAP-25/GFP constructs discussed in this paper are summarized in Fig. 1. Standard molecular cloning techniques were used to manipulate DNA (20). The integrity of all constructs derived from PCR products was verified by DNA sequence analysis. Plasmids pEGFP-N and pEGFP-C, which fuse the GFP coding region to the 3′- or 5′-end of the inserted sequence, respectively, were purchased from CLONTECH (Palo Alto, CA). Dr. Michael C. Wilson (University of New Mexico) kindly provided the SNAP-25b cDNA. Full-length SNAP-25 was inserted into pEGFP-C as a *KpnI/Bam*HI fragment (GFP/1–206). 1–142/GFP was first introduced into pEGFP-N as a *KpnI/Sma*I fragment. To eliminate the 5′-untranslated region in 1–142/GFP, we generated a 5′-oligonucleotide that introduces a *Xho*I site and anneals to the first 18 bases of the SNAP-25 open reading frame. The PCR product encoding residues 1–142 of SNAP-25 was introduced into pEGFP-N as a *XhoI/Sma*I fragment. PCR products encoding residues 1–115 and 1–120 of SNAP-25 were cloned into pEGFP-N as *XhoI/Bam*HI fragments. SNAP-25 residues 45–142 and 56–142 were inserted into pEGFP-N as *XhoI/Sma*I fragments. The constructs 56–95/GFP and 1–95/GFP were generated using a 3′-reverse oligonucleotide annealing across the unique *Hind*III site of the SNAP-25 coding region and 5′-oligonucleotides annealing at appropriate start sites. The PCR products were then introduced into pEGFP-N as *BglII/Hin*dIII (56–95/GFP) and *XhoI/Hin*dIII (1–95/GFP) fragments. The PCR product encoding residues 85–120 was cloned into pEGFP-N as a *XhoI/Bam*HI fragment. An in-frame ATG codon was included in the 5′-oligonucleotide primers for SNAP-25 constructs with N-terminal deletions.

To replace all four cysteine residues with alanines in 1–142/GFP, a two-step construction was used. First, residues 85 and 88 were mutated using a PCR-based strategy. A PCR product was generated using a 5′-oligonucleotide corresponding to the first 18 bases of the SNAP-25 open reading frame and a mutant 3′-reverse oligonucleotide that annealed across the unique *Hin*dIII site of the SNAP-25 coding sequence. The plasmid 1–142/GFP was digested with *Hin*dIII and *Xho*I to generate a vector containing 95–142/GFP. The C85–88A PCR fragment (*XhoI/Hin*dIII) was ligated with the vector. The resulting plasmid was used as template to mutate cysteine residues 90 and 92, using a similar strategy. Gln^{193}, Pro^{197}, and Arg^{198} of SNAP-25 were mutated to alanine in the context of the 85–120/GFP fusion using the Quikchange mutagenesis kit (Stratagene, La Jolla, CA).

Transient Transfection of NG108 Cells—NG108 cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 150 units/ml penicillin, and 50 μg/ml streptomycin. The cells were grown in six-well or 35-mm tissue culture plates coated with poly-L-lysine until 50–80% confluent. Cells were transfected using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions and analyzed 24 h later.

Confocal Laser Microscopy—Twenty hours after transfection, NG108 cells were washed with phosphate-buffered saline and fixed with freshly prepared 4% (w/v) paraformaldehyde for 15 min at room temperature. Subsequently, cells were washed with phosphate-buffered saline, and coverslips were mounted on slides with a drop of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Cells were examined using a Zeiss Axioplan microscope coupled to an MRC-1000 laser scanning confocal microscope (Bio-Rad) with a 63× oil immersion objective. Confocal images were assembled as montages using Adobe Photoshop Version 3.0 and Canvas 3.5

Radiolabeling, BFA Treatment, and Immunoprecipitation—Cells were radiolabeled with [^{35}S]methionine and [^{3}H]palmitate for 90 min as described (15). Brefeldin A suspended in dimethyl sulfoxide was added to the cells along with the radiolabel (final concentration of 10 μg/ml). Control cells were incubated with dimethyl sulfoxide alone. Cells were solubilized with radioimmune precipitation assay buffer and processed for immunoprecipitation as described (15). Lysates were immunoprecipitated with rabbit anti-GFP polyclonal antibody (CLONTECH). Radiolabeled polypeptides were detected by fluorography. [^{35}S]Methionine-labeled proteins were detectable after 2 or 3 h, and [^{3}H]palmitate-labeled proteins after overnight exposure.

Subcellular Fractionation—Cells growing in 100-mm dishes were transfected with the appropriate constructs and, 16–20 h after transfection, were washed with warm phosphate-buffered saline and scraped into ice-cold phosphate-buffered saline. Cells were collected by centrifugation and suspended in a hypotonic buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, and 10 μg/ml pepstatin). After a 15-min incubation, cells were homogenized with 20 passes through a ball-bearing homogenizer and pelleted at 800 × *g* for 5 min. The pellet consisting of unbroken cells and nuclei was designated P1. The post-nuclear supernatant was centrifuged at 100,000 × *g* for 30 min. The resulting supernatant (S100 fraction) was mixed with an equivalent volume of 2× radioimmune precipitation assay buffer. The 100,000 × *g* pellet (P100 fraction) was solubilized by suspension in radioimmune precipitation assay buffer. Equal fractions of P1, P100, and S100 were analyzed by immunoblotting. GFP fusion proteins were detected with an anti-GFP monoclonal antibody (CLONTECH) and ^{125}I-labeled secondary antibody (ICN, Costa Mesa, CA). Quantitation of the immunoblots was performed using a PhosphorImager screen and ImageQuant software (Molecular Dynamics, Inc.).

RESULTS

Full-length SNAP-25 Targets GFP to the Plasma Membrane—SNAP-25 is localized predominately at the plasma membrane in neurons and neuronal cell lines (21, 22). To determine if GFP could be used as a reporter to characterize the membrane-targeting domain of SNAP-25, we constructed a fusion protein with full-length SNAP-25 fused to GFP (Fig. 1). This construct was transiently transfected into NG108 cells, and its localization was visualized by fluorescence microscopy. As shown in Fig. 2*A*, GFP alone localized to the cytosol of NG108 cells. In contrast, the SNAP-25/GFP fusion was localized at the plasma membrane and enriched in cellular processes. This pattern is very similar to that of endogenous protein and indicates that full-length SNAP-25 can direct GFP to the plasma membrane. Immunoblot analysis confirmed that the fusion protein was the appropriate molecular mass (Fig. 2*B*).

Residues 85–120 of SNAP-25 Target GFP to the Plasma Membrane—To identify the minimal plasma membrane-targeting elements within SNAP-25, we constructed a series of deletion mutants of SNAP-25 fused to GFP (Fig. 1). SNAP-25 contains two sets of heptad repeats at the N terminus interrupted by a break in frame (residues 1–42 and 45–90) (23). A third set of heptad repeats is found in the C-terminal region of the protein between residues 157 and 205. These heptad repeats form α-helices that are involved in intermolecular coiled-coils (4). We began the analysis by deleting the C-terminal set of heptad repeats (*H3* in Fig. 1) and expressed a protein with the N-terminal 142 amino acids of SNAP-25 fused to GFP. As shown in Fig. 3*a*, amino acids 1–142 of SNAP-25 were able to target GFP to the plasma membrane, indicating that the C-terminal portion of the protein is not required for proper tar-

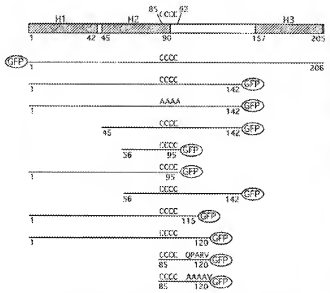

FIG. 1. **Schematic representation of fusion proteins used in this study.** The top diagram represents full-length SNAP-25. *Hatched bars* indicate α-helical domains composed of sets of heptad repeats (*H1*, *H2*, and *H3*). The cysteine-rich domain between residues 85 and 92 is shown in all constructs. The *numbers* on the fusion proteins indicate the amino acids of the human SNAP-25b isoform that were fused to GFP.

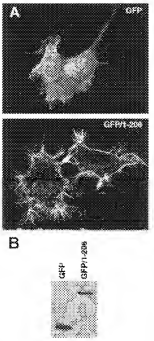

FIG. 2. **SNAP-25/GFP fusion protein (GFP/1–206) is targeted to the plasma membrane.** NG108 cells transfected with GFP or full-length SNAP-25 fused to GFP (GFP/1–206) were fixed and visualized by fluorescence microscopy (*A*) or lysed and analyzed by immunoblotting with an anti-GFP monoclonal antibody and detected using enhanced chemiluminescence (*B*).

geting. As for the full-length protein (18, 19), plasma membrane localization of the 1–142/GFP chimera depended on the four palmitoylated cysteine residues since a mutant chimeric protein in which the four cysteines were mutated to alanines had a cytoplasmic distribution (Fig. 3*b*).

To further characterize the membrane-targeting domain of SNAP-25, we constructed a truncated chimeric protein lacking the most N-terminal set of heptad repeats (45–142/GFP) (*H1* in Fig. 1). Based on studies of *in vitro* binding reactions of

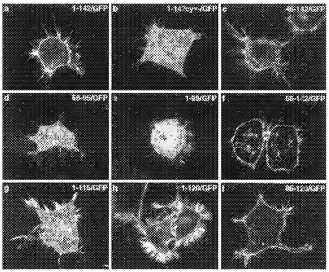

FIG. 3. **Subcellular distribution of deletion mutants of SNAP-25/GFP.** NG108 cells were transfected with SNAP-25 deletion mutants and visualized by fluorescence microscopy. Note that residues 85–120 constitute the minimal domain of SNAP-25 necessary and sufficient to target GFP to membranes (*i*).

SNAP-25 deletion mutants with syntaxin, the loss of residues 1–44 should eliminate SNAP-25 interactions with syntaxin (23). This fusion protein localized to the plasma membrane of NG108 cells (Fig. 3*c*), indicating that SNAP-25 can associate with membranes independently of interactions with syntaxin.

Residues 45–142 of SNAP-25 include the amino acids encoded by the alternatively spliced exon 5 of the gene (amino acids 56–95). The two isoforms of SNAP-25 (a and b) differ at nine residues within this region (24), and it has been proposed that these differences might confer differential localization of the two isoforms (25). We tested whether exon 5 encodes all of the membrane-targeting information by fusing residues 56–95 to GFP. As shown in Fig. 3*d*, sequences encoded by exon 5 were not sufficient to target GFP to membranes. Extension of this region at the N terminus (1–95/GFP) did not restore membrane localization (Fig. 3*e*). However, extension of this region at the C terminus (56–142/GFP) resulted in an appropriate plasma membrane distribution (Fig. 3*f*). Thus, residues 1–55 were dispensable for targeting, but some residues between positions 95 and 142 were required for plasma membrane localization.

To define the C-terminal boundary of the membrane-targeting domain of SNAP-25, we made serial deletions inward from residue 142. The constructs 1–115/GFP and 1–120/GFP revealed that the N-terminal 115 amino acids were not sufficient to target GFP to the plasma membrane (Fig. 3*g*), but residues 1–120 were (Fig. 3*h*). Using residue 120 as the C-terminal boundary, we established the N-terminal boundary of the membrane-targeting domain by making sequential deletions from residues 56 to 85, the position of the first palmitoylated cysteine. As shown in Fig. 3*i*, amino acids 85–120 of SNAP-25 were sufficient to target a heterologous protein to the plasma membrane of NG108 cells. Interestingly, this sequence coincides almost exactly with the protease-sensitive interhelical domain (residues 83–120) of SNAP-25 in the SNARE complex (11, 12). All of the deletion constructs were expressed at similar levels and migrated at the predicted molecular mass as assessed by immunoblotting (data not shown). The N-terminal boundary of the membrane-targeting domain was defined as residue 85 because mutation of this site in the context of the full-length protein (or in 1–142/GFP) results in a significant decrease in

membrane association and loss of radioactive palmitate incorporation (18).[2]

Palmitoylation of the Membrane-targeting Domain of SNAP-25 Is Sensitive to BFA—To investigate if the chimera 85–120/GFP retains the ability to be palmitoylated, we incubated NG108 cells expressing 85–120/GFP with [^{3}H]palmitate and assayed for incorporation of radiolabel into the immunoprecipitated protein. As shown in Fig. 4, the 85–120/GFP protein (*lane 7*) incorporated [^{3}H]palmitate, as did the GFP fusion with the full-length sequence of SNAP-25 (*lane 3*). Palmitoylation and membrane association of newly synthesized endogenous SNAP-25 require an intact secretory pathway in neuronal cell lines (PC12, N2A, and NG108 cells) (15). To determine if palmitoylation of the membrane-targeting domain of SNAP-25 resembles that of endogenous protein, we evaluated the sensitivity of this process to BFA. As shown in Fig. 4, palmitoylation of both full-length SNAP-25 (*lane 4*) and the membrane-targeting domain (*lane 8*) was inhibited by BFA. The inhibitory effect of BFA on palmitoylation was not due to decreased protein expression, as equal amounts of [^{35}S]methionine-labeled protein are found in the immunoprecipitates in the presence or absence of drug (*lanes 1, 2, 5,* and *6*). Our results indicate that the post-translational processing of the membrane-targeting domain of SNAP-25 (residues 85–120) is similar to that of endogenous SNAP-25.

A Conserved Sequence of Five Amino Acids Is Necessary for Efficient Localization and Palmitoylation of the Membrane-targeting Domain—Analysis of the deletion mutants revealed that residues 116–120 are required for localization of SNAP-25 at the plasma membrane. Alignment of the sequences of SNAP-25 family members with residues 85–120 of SNAP-25b (Fig. 5) revealed that three of the five residues are absolutely conserved in all of the sequences. To determine if the conserved residues are important for membrane targeting, we made alanine substitutions at Gln^{116}, Pro^{117}, and Arg^{119} in 85–120/GFP (85–120 QPR/GFP). As shown in Fig. 6, 85–120 QPR/GFP was poorly localized at the plasma membrane (*center panel*). Although some plasma membrane fluorescence was evident, there was prominent cytoplasmic staining when compared with the intact membrane-targeting domain (*left panel*). However, mutation of the three residues did not inhibit localization as severely as did deletion of all five residues (*right panel*).

We performed subcellular fractionation studies to confirm the ability of residues 85–120 to facilitate membrane interactions. We analyzed the distribution of 85–120/GFP, 1–115/GFP, and 85–120 QPR/GFP in particulate and soluble fractions of transfected NG108 cells. 85–120/GFP was found predominately in the P100 (membrane) fraction, whereas both 85–120 QPR/GFP and 1–115/GFP were predominately cytosolic (Fig. 7). The results of the subcellular fractionation confirm the localization depicted by fluorescence microscopy.

Palmitoylation of the mutated proteins correlated with membrane association. The amount of [^{3}H]palmitate incorporated into 85–120 QPR/GFP and 1–115/GFP was substantially reduced compared with intact 85–120/GFP (Fig. 8). Although the cysteine residues are maintained in both 85–120 QPR/GFP and 1–115/GFP, these data suggest that the five-amino acid motif QPARV facilitates palmitoylation of the protein, thereby promoting membrane association of SNAP-25.

DISCUSSION

In this study, we mapped the minimal region of SNAP-25 that confers targeting specifically to the plasma membrane to residues 85–120. The membrane-targeting domain represents two-thirds of the interhelical loop that connects the N- and C-terminal α-helices of SNAP-25 in the SNARE complex (Fig. 9). We have established that an important function of the interhelical loop is to localize SNAP-25 at the plasma membrane. This function appears to be independent of SNARE interactions since removal of the regions of SNAP-25 that form coiled-coils with syntaxin and synaptobrevin did not interfere with proper targeting of SNAP-25 fusion proteins. It is noteworthy that the membrane-targeting domain coincides almost exactly with the protease-sensitive region of SNAP-25 (residues 83–120) in the SNARE complex. The sensitivity of SNAP-25 residues 83–120 in the complex to proteases (11, 12) and the fact that this domain had to be deleted to obtain crystals of the core SNARE complex suggest that it may be disordered *in vitro*. However, it seems probable that interactions with the bilayer or with other proteins will result in a more ordered structure of the membrane-binding domain.

Residues 85–120 of SNAP-25 constitute a membrane-targeting domain with unique characteristics. Plasma membrane association not only is dependent on the stretch of palmitoylated cysteines contained within the first eight amino acids of the targeting sequence (Ref. 18 and this study), but also requires an additional 27 amino acids. This distinguishes

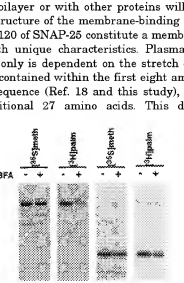

FIG. 4. **Palmitoylation of SNAP-25/GFP fusion proteins is sensitive to BFA.** NG108 cells transfected with GFP/1–206 or the membrane-targeting domain 85–120/GFP were incubated with [^{3}H]palmitate (*[^{3}H]palm; lanes 3, 4, 7,* and *8*) or [^{35}S]methionine (*[^{35}S]meth; lanes 1, 2, 5,* and *6*) for 90 min in the presence or absence of 10 μg/ml BFA. The incorporation of radiolabel into the immunoprecipitated proteins was assessed by SDS-polyacrylamide gel electrophoresis and fluorography. Note how palmitoylation of both chimeric proteins was sensitive to treatment with BFA. Protein expression, however, was not affected by BFA treatment.

[2] S. Gonzalo and M. E. Linder, unpublished results.

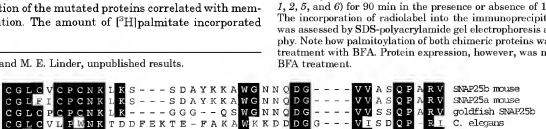

FIG. 5. **Sequence alignment of SNAP-25 family members.** Sequences corresponding to the membrane-targeting domain (residues 85–120) of the SNAP-25b isoform from SNAP-25 family members were aligned using the Clustal method (DNASTAR, Inc.). Residues conserved in at least six of eight sequences are *boxed*. GenBank™ accession numbers for the sequences are as follows: mouse, M22012; goldfish, 548945; *Torpedo*, L22020; *Drosophila*, L22021; *Caenorhabditis elegans*, 3880712; human SNAP-23, U55936; and mouse syndet, U73143.

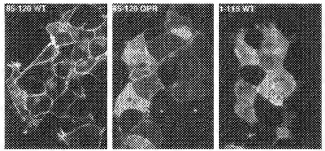

FIG. 6. **Plasma membrane localization of the interhelical domain of SNAP-25 requires amino acids 116–120.** NG108 cells were transfected with 85–120/GFP (*left panel*), 85–120 QPR/GFP (*center panel*), or 1–115/GFP (*right panel*) and visualized by confocal microscopy. Deletion of residues 116–120 or mutation of three of the five residues results in significant reduction of plasma membrane staining of SNAP-25 fusion protein. *WT*, wild type.

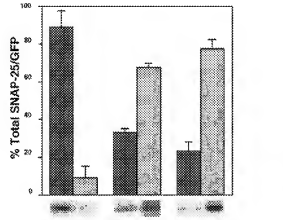

FIG. 7. **Residues 116–120 of SNAP-25 are important for membrane association.** Shown are the results from immunoblot analysis of subcellular fractions of NG108 cells expressing 85–120/GFP (*left bars*), 85–120 QPR/GFP (*center bars*), or 1–115/GFP (*right bars*). The distribution of SNAP-25 fusion protein in P100 (*dark bars*) and S100 (*light bars*) fractions was quantitated using a PhosphorImager. Data are expressed as the mean ± S.D. of three (85–120 QPR/GFP and 1–115/GFP) or four (85–120/GFP) experiments. A representative experiment is shown below the graph. Not shown are the P1 fractions (low speed pellet) that contained approximately one-fourth of the immunoreactivity for all constructs. Only the intact interhelical domain of SNAP-25 (residues 85–120) was found predominately in the particulate fraction.

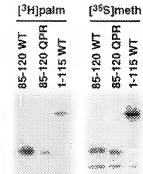

FIG. 8. **Residues 116–120 facilitate palmitoylation of the interhelical domain of SNAP-25.** SNAP-25 fusion proteins were analyzed for incorporation of [^{3}H]palmitate (*^{3}H/palm; left panel*) or [^{35}S]methionine (*^{35}S/meth; right panel*) as described under "Experimental Procedures." 85–120/GFP labeled with [^{35}S]methionine migrates as a doublet because palmitoylation of the protein results in a shift in electrophoretic mobility (15). Deletion or mutation of residues 116–120 significantly reduced incorporation of [^{3}H]palmitate into the interhelical domain of SNAP-25. *WT*, wild type.

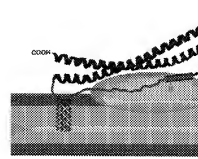

FIG. 9. **Model of membrane interactions of SNAP-25.** The N- and C-terminal α-helices of SNAP-25 that interact with syntaxin and synaptobrevin are shown as *dark-gray coils*. The membrane-targeting domain (residues 85–120) is shown binding to a hypothetical membrane protein (*light-gray oval*). Residues 116–120 are shown as a *cylinder* at the end of the membrane-targeting domain. Palmitate groups attached to cysteines 85, 88, 90, and 92 are depicted inserting into the lipid bilayer. The loop (residues 121–137) connecting the C terminus of the membrane-targeting domain with the second α-helix is shown in *black*. (adapted with permission from Sutton *et al.* (4)).

SNAP-25 from other lipid-modified proteins in which the boundary of the membrane-targeting sequence is in close proximity to lipid-modified amino acids. Targeting information for non-receptor tyrosine kinases p59^{fyn} and p56^{lck} is contained in their 10 N-terminal residues (26, 27). This region includes the myristoylated glycine at position 2 and two nearby cysteines modified by palmitate. Membrane association of p59^{fyn} is maintained when this sequence is replaced with the first 10 amino acids of GAP-43, which includes two palmitoylated cysteines (14).

There are two distinct regions of highly conserved sequence across species that can be observed when the predicted membrane-targeting domains of SNAP-25 family members are aligned (Fig. 5). These regions coincide with the N- and C-terminal boundaries of the membrane-targeting domain. The first is the cysteine-rich region (residues 85–92). The second is a five-amino acid motif (QPXR(V/I)) at the C terminus of the domain. Mutation of three of the five amino acids (Gln, Pro, and Arg) to alanine severely compromised localization of the membrane-targeting domain and significantly decreased palmitate incorporation. SNAP-25 is synthesized as a soluble protein, but must associate with a membrane-bound palmitoyltransferase to become fatty acylated (15). Thus, there must be a mechanism to provide at least transient interaction of SNAP-25 with membranes to permit palmitoylation. We speculate that the C-terminal QPARV motif permits binding of SNAP-25 to a membrane protein, thereby facilitating recognition of SNAP-25 by a membrane-bound palmitoyltransferase (Fig. 9). It is possible that the same molecule displays both functions, *i.e.* binding to the membrane-targeting domain and palmitoylation.

A requirement for membrane association prior to palmitoylation has been observed for a number of palmitoylated proteins. Modification of proteins at tandem lipidation motifs occurs sequentially (reviewed in Ref. 13). Palmitoylation of non-receptor tyrosine kinases is dependent upon prior myristoylation. Similarly, prenylation is a prerequisite for palmitoylation of Ha-Ras and N-Ras. These proteins are modified with an *N*-myristoyl or farnesyl group in the cytoplasm. The addition of the first lipid provides a low affinity interaction with membranes that appears to permit contact with the palmitoyltransferase. The addition of the second lipid significantly increases membrane affinity, resulting in essentially permanent association of the protein on the membrane where it was modified with palmitate (28). G-protein α-subunits that are dually modified with myristate and palmitate exhibit a similar

interdependence of lipid modifications. However, the failure to palmitoylate myristoylation-defective Gα subunits can be overcome by coexpressing G-protein βγ-subunits (29, 30). Thus, there is precedent for interaction of a palmitoyltransferase substrate with a membrane protein other than a palmitoyltransferase prior to its modification.

Our finding that the interhelical domain of SNAP-25 confers membrane localization raises the possibility that this region functions similarly in other SNAP-25 family members. Morphological analysis of the yeast exocytic complex confirms that Sec9p, a yeast SNAP-25 homolog, forms a four-helix bundle with the yeast syntaxin homolog Sso1 or Sso2 and synaptobrevin homolog Snc1 or Snc2 (31). Plasma membrane localization of Sec9p *in vivo* does not require Sso1/2 (32), suggesting that its membrane localization may be independent of SNARE interactions. Sec9p lacks palmitoylation sites; thus it must associate with membranes through mechanisms other than fatty acylation.

SNAP-23 and syndet are ubiquitously expressed isoforms of SNAP-25 that are localized at the plasma membrane (33, 34). In contrast, a newly identified family member, SNAP-29, is localized predominantly on intracellular membranes when transfected into normal rat kidney cells (35). Its distribution overlaps with that of markers for the Golgi, the *trans*-Golgi network, and endosomal compartments. Sequences directing membrane attachment of SNAP-23 and SNAP-29 have not been well characterized. The cysteine-rich sequence within the membrane-targeting domain of SNAP-25 is conserved in SNAP-23 and syndet as well as the QPXR(V/I) motif (Fig. 5). The sequence similarity strongly suggests conservation of the membrane-targeting function of the interhelical domain. Baldini and co-workers (36) have recently reported that plasma membrane localization of syndet requires the cysteine-rich domain (36). Interestingly, residues 89–101 (RTKNFES-GKNYKA) of syndet are dispensable for plasma membrane localization (36). These residues immediately follow the cysteine stretch in syndet and correspond to a part of the membrane-targeting domain of SNAP-25 that is poorly conserved among family members (Fig. 5).

The putative interhelical domain of SNAP-29 lacks palmitoylation sites and has no other sequence similarity to SNAP-25, SNAP-23, and syndet. However, a significant fraction of SNAP-29 behaves as an integral membrane protein when expressed in COS cells. It has been suggested that SNAP-29 is recruited to membranes through interactions with different syntaxins and vesicle-associated membrane proteins, bypassing the need for a strict membrane-anchoring motif and allowing it to function in a variety of compartments (35). We have demonstrated that SNAP-25 can be targeted to the plasma membrane independently of its interactions with SNARE proteins. However, this does not exclude the possibility that a SNARE-dependent mechanism also exists. It will be important to define the mechanisms of membrane association of SNAP-25 family members to test whether the interhelical domain has a general role in membrane localization or serves a specialized function for neuronal SNAP-25.

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